Integrin Targeting and Toxicological Assessment of Peptide-Conjugated Liposome Delivery Systems to Activated Endothelial Cells

Integrin targeting and toxicological assessment of peptide-conjugated liposome delivery systems to activated endothelial cells

Ali Kermanizadeh[1], Klaus Villadsen[2], Ragnhild G. Østrem[3], Knud J. Jensen[2], Peter Møller[1] and Steffen Loft[1]

[1]University of Copenhagen, Department of Public Health, Section of Environmental Health, Copenhagen, Denmark, [2]University of Copenhagen, Biomolecular Nanoscale Engineering Center (BioNEC), Department of Chemistry, Copenhagen, Denmark, [3]Technical University of Denmark, Colloids and Biological Interfaces Group, Department of Micro- and Nanotechnology, Center for Nanomedicine and Theranostics, Lyngby, Denmark

Author for correspondence: Peter Møller, University of Copenhagen, Department of Public Health, Section of Environmental Health, Øster Farimagsgade 5A, Copenhagen, 1014, Denmark

e-mail: pemo@sund.ku.dk

Running title: Integrin targeting of peptide modified liposomes

Abstract

Utilisation of functionalized liposomes as the means of targeted delivery of therapeutics may enhance specific transport of biologically active drugs to target tissues, while avoiding or reducing undesired side-effects. In the present investigation, peptide-conjugated cationic liposomes were constructed with the aim of targeting integrins (i.e. vitronectin and/or fibronectin receptors) on activated endothelial cells. The peptide-conjugated liposomes induced only cytotoxicity at the highest concentration in non-activated or activated endothelial cells, as well as in co-culture of endothelial cells and macrophages. There was unaltered secretion of cytokines following exposure of peptide-conjugated liposomes to endothelial cells, indicating that the materials were not inflammogenic. Liposomes with a peptide targeting the fibronectin receptor (integrin $\alpha 5\beta 1$) were more effective in targeting of activated endothelial cells, as compared to a liposome with a peptide that targeted both the fibronectin and vitronectin receptors, as well as liposomes with a control peptide. The liposome targeted to the fibronectin receptor also displayed uptake in endothelial cells in co-culture with activated macrophages. Therefore, this study demonstrates the feasibility of constructing a peptide-conjugated cationic liposome, which displays targeting to activated endothelial cells at concentrations that are not cytotoxic or inflammogenic to the cells.

Introduction

Liposomes are artificial vesicles made of an aqueous core surrounded by a lipid bilayer [1]. These vesicles have been designed and utilised as delivery vehicles for drugs, genetic material and imaging agents for parental administration [2]. The encapsulation of the cargo into the liposomes can protect the drug against metabolic transformation, enable transport across biological barriers, and control the release in target tissues, hence the subsequent alleviation of side effects associated with drug. Due to their small size, charge and the possibility for modification and inclusion of targeting moieties, liposomes can be ideal candidates for improved targeting and delivery to the target tissue [3]. Indeed, liposomes can accommodate a wide range of specific adhesion molecules and polymers on their surface, which can aid adhesion to vascular cells [4-8]. Furthermore, it has been demonstrated that liposomes exhibit enhanced permeability and retention in certain areas of the vasculature, which suggests that it is possible to influence the distribution of liposomes in the cardiovascular system [9].

Endothelial cells line all blood vessels and regulate the flow of nutrients, biologically active molecules and an array of leukocytes. This role of the endothelium is governed through membrane-bound receptors, lipid transporting particles, hormones and proteins that govern cell-cell and cell-matrix interactions [10]. Integrins (i.e. αv integrins) on the endothelial cells bind to extracellular matrix proteins and other adhesion receptors on neighbouring cells. The integrins on the surface of a cell will determine whether it can adhere to and/or survive in a particular microenvironment, therefore the matching of integrins and ligands play a key role in the regulation of the sprouting ability of endothelial cells during angiogenesis and localization of leukocytes to sites of tissue inflammation. The peptide sequences arginine-glycine-aspartic acid (RGD) and C16Y (DFKLFAVYIKYR) have been identified as ligands for interaction and binding of integrins αvβ3, αvβ5 and α5β1. The usefulness of these sequences has been exploited as drug delivering systems to

target specific cell types [11-14]. The challenge is to attach a short peptide to liposomes that specifically target the cell type in question for therapy. The integrin α5β1 (fibronectin receptor) and αvβ3 (vitronectin receptor) are typically expressed on endothelial cells, although the number of receptors depends on the type of endothelial cells. For instance, the fibronectin receptor is much more abundant on primary human umbilical vein endothelial cells (HUVECs) than the vitronectin receptor [15, 16]. The vitronectin receptor is abundantly expressed on angiogenic endothelial cells in remodelling and pathological tissues as compared to the expression in normal quiescent endothelial cells [17]. The vitronectin and fibronectin receptors are ubiquitous integrins, playing an important role in a diverse range of biologic processes including cell migration, tumour invasion, angiogenesis and immune responsiveness [18-21].

To date, very few studies have designed stable, novel peptide-conjugated liposomes for potential cardiovascular disease therapy (with negligible adverse immunogenic capacity as well as enhanced targeting ability). In the present investigation, we investigated the targeting and toxicity of cationic liposomes with functionalization to target integrins on activated endothelial cells. We assessed differences in targeting and toxicity in endothelial cells that were in normal state and after insults such as local inflammation and early atherosclerotic lesions: 1) quiescent endothelial cells, 2) endothelial cells activated by lipopolysaccharide (LPS), 3) activated cells with presence of immune cells (e.g. activated macrophages or foam cells). The co-culture model was utilized to mimic the inflammatory response when phagocytes (predominantly macrophages) increase adhesiveness to the endothelium, secrete inflammatory soluble proteins and produce reactive oxygen species. Activated macrophages engulf materials through binding to scavenger receptors. Thus, they may compete with endothelial cells with regards to uptake of peptide-modified liposomes.

Materials and methods

Synthesis of unconjugated liposomes

The lipids 1,2-dioleoyl-sn-glycero-3-phoshocholine (DOPC) (Avanti Polar Lipids Inc., Alabaster, AL, USA) and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) (Avanti Polar Lipids Inc., Alabaster, AL, USA) (molar ratio - 9:1) were dissolved in chloroform and thoroughly mixed in a glass vial. The solution was allowed to dry under vacuum for 2 hr in order for a lipid film to form on the glass and to ensure the complete removal of chloroform. The unconjugated liposomes were rehydrated carefully by the addition of phosphate buffered saline (PBS), for a final lipid concentration of 1 mg/ml. The mixture was incubated unstirred overnight at room temperature. The following day, the unconjugated liposomes were subjected to ten freeze-thaw cycles to minimize multi-lamellarity by immersion in liquid nitrogen, followed by thawing in 40ºC water bath. The liposomes were sequentially extruded through two stacked polycarbonate filters with pore sizes of 50 nm (Mini-extruder - Avanti Polar Lipids Inc., Alabaster, AL, USA), before being stored at 4ºC until use.

Peptide design and synthesis

The lipidated peptides for targeting integrins were assembled by solid-phase peptide synthesis (SPPS) using amino acids carrying an Nα-9-fluorenylmethyloxy carbonyl (Fmoc) protecting group (see Supplementary Information). The lipid chain was introduced by on-resin modification of a C-terminal Lys (fig. 1). This Lys residue was incorporated with a allyloxycarbonyl (Alloc) side-chain protecting group, as Fmoc-Lys(Alloc)-OH). After assembly of the linear sequence, the Alloc protecting group was selectively removed, while the other protecting groups remained intact. The free Lys side-chain amine was acylated with palmitic acid to ensure optimal anchoring to the liposome surface. Next, the N-terminal Fmoc protecting group was removed and the N-terminal

amine tagged by amide bond formation with an ATTO465 fluorophore for *in vitro* fluorescent detection. Finally, the peptides were deprotected and released from the support.

Peptides **2** and **3** were designed to target integrins αvβ3, αvβ5 and/or α5β1 on endothelial cells while peptide **1** was designed as randomized control sequence of amino acids (fig. 2).

Preparation of the peptide-conjugated liposomes

The three different peptides were added to the liposomes (1:5 weight ratio) in PBS and incubated unstirred overnight at room temperature in containers that were wrapped in tinfoil to avoid contract with light. Peptide-conjugated liposomes were then separated from unbound peptides using a PD-10 desalting column according to manufacturer's instructions (GE Healthcare, Brøndby, Denmark).

Characterisation of unconjugated liposomes

The hydrodynamic size distributions of the unconjugated liposomes dispersed in filtered water, PBS or complete cell culture medium was determined in the 10-50 µg/ml concentration range by Nanoparticle Tracking Analysis (Nanosight LM20, UK). The zeta potential of the liposomes in PBS were measured in a standard Malvern disposable folded capillary cells in a Zetasizer nano ZS with Malvern version 6.20 Software. A limulus amebocyte lysate (LAL) Pyrogent™ Plus assay (Lonza, Basel, Switzerland) was utilised to test for possible endotoxin contaminations of the liposomes. The kit was used according to the manufacturer's guidelines.

Cell culture and liposome treatment

Primary HUVECs (Cell Applications, San Diego, CA, USA) were cultured in T75 flasks in endothelial cell growth medium (Cell Applications, San Diego, CA, USA). All incubations were carried out at 37°C and 5% CO_2. The cells were utilised between passages 2-10 as they retain

morphologic and phenotypic characteristics of endothelial cells. THP-1 monocytes (American Type Culture Collection, Manassas, VA, USA) were cultured in Roswell Part Memorial Institute (RPMI) medium (Gibco, The Netherlands) supplemented with 10% foetal bovine serum (FBS) (Gibco, The Netherlands), 2 mM L-glutamine and 100 U/ml Penicillin/Streptomycin (Sigma, UK).

The HUVECs were activated utilising 2 µg/ml of *Escherichia coli* LPS for 2 hr (serotype O26:B26, Sigma, UK). In the co-culture model, the HUVECs and THP-1 cells (4:1 ratio) were cultured in complete HUVEC medium supplemented with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma, UK) 24 hr before the addition of LPS for 2 hr [22]. The cells were rinsed in PBS and subsequently treated with liposomes.

The cell cultures were exposed to liposomes in a concentration range between 0.61 and 312.5 µg/cm^2 (equivalent to 2-1000 µg/ml).

WST-1 cell viability assay

The cells (all three different cultures - non-activated HUVECs, activated HUVECs and HUVECs/macrophage co-culture) were seeded in 96 well plates (10^4 cells per well in 100 µl of the cell culture medium) and incubated for 24 hr before they were exposed to the liposomes for 24 hr. The first 24 hr incubation period was used to avoid a seeding effect in the cells and the subsequent 24 hr period is a standardized incubation for measurement of cytotoxicity with the WST-1 assay. Subsequent to treatment with peptide-conjugated liposomes, cell supernatants were collected and frozen at -80ºC and later used for soluble protein measurements. The plates were incubated with 10 µl of the WST-1 cell proliferation reagent (Roche, USA) and 90 µl of fresh medium for 1 hr at 37ºC and 5% CO_2. The supernatant was transferred to a fresh plate and the absorbance was measured by spectrophotometry at 450 nm (the supernatants transfer into fresh plates was carried out to reduce the potential interference with materials during the measurements).

Peptide-conjugated liposome-induced inflammatory response

After exposure, the cell culture supernatants were collected and stored at -80ºC. The concentrations of interleukin (IL) 1β, IL6, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), macrophage inflammatory protein-1α (MIP-1α), tumour necrosis factor-α (TNF-α) and granulocyte-colony stimulating factor (G-CSF) supernatants were determined with BD^{TM} Cytometric Bead Array cytokine flex sets (bead based immunoassay; BD Biosciences, USA). Flow cytometry was utilised to discriminate between different bead populations based on size and fluorescence, according to the manufacturer's instructions. The flex sets employ microparticles with distinct fluorescence intensities to detect soluble analytes (in this case cytokines/chemokines). In these experiments no positive control was included as we do not have a compound that increases the entire set of markers in both THP-1 cells and HUVECs. LPS exposure only increases the secretion of some cytokines and it is not the same concentration that produces a maximal response of different cytokines (unpublished observations). We have regarded the substantial difference in cytokine release between the non-activated HUVECs, activated HUVECs and co-culture as sufficient evidence of ability of the flex sets to determine concentration-response relationships in cultured cells.

Liposome uptake - fluorescent microscopy

The cells were seeded in 8 well microscopy chambers (Ibidi, Germany) (10^4 cells per well in 200 µl of the cell culture medium) and incubated for 24 hr at 37ºC and 5% CO_2. The cells were exposed to PMA and/or LPS as previously described. The cells were exposed to the different peptide-conjugated liposomes for 2 hr (7.8, 15.6 and 31.25 µg/cm^2). Following liposome exposure, the cells were washed thoroughly with PBS and observed under a Leica AF6000 inverted wide-field

fluorescence microscope (Leica, Germany). The 2 hr exposure period for the microscopic uptake studies was based on preliminary data from time-course experiments utilising liposomes with similar physico-chemical characteristics (unpublished data) and previously published work with gold nanoparticles [23, 24]. The experiments were carried out on three different days; only representative images of 15.6 µg/cm^2 are shown in the paper.

Flow cytometric analysis of cell-associated fluorescence

Following a 2 hr peptide-conjugated liposome treatment (15.6 µg/cm^2), cell cultures (as described above) (10^6 cells per well) were washed three times and detached using trypsin/EDTA. The cells were centrifugated and re-suspended in 200 µl of PBS and analysed by flow cytometry using an Accuri C6 flow cytometer (Becton Dickinson, USA). The results are reported as the mean fluorescence from 10^4 cells, which were analysed in each flow cytometry analysis.

Semi-quantification of cellular uptake

The cells (all three different cultures) were seeded in 96 well plates (10^4 cells per well in 100 µl of the cell culture medium) and incubated for 24 hr at 37ºC and 5% CO_2. The following day the cells were exposed to the different peptide-conjugated liposomes (15.6 µg/cm^2) for 2 or 6 hr at 37ºC and 5% CO_2. The supernatant from each well was transferred to opaque plates and the fluorescence was measured (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Scientific, USA).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). For statistical analysis, the experimental results were compared to their corresponding control values using full-factorial

206 ANOVA with Tukey's multiple comparison. All statistical analysis was carried out utilizing
207 Minitab 17. All experiments were repeated independently on at least three separate occasions.
208
209 **Results**
210 *Liposome characterisation*
211 The particle sizes of the unconjugated liposomes were determined in filtered water, PBS and the
212 HUVEC complete medium (table 1). Representative examples of the particle size distribution are
213 provided in supplementary figure 1. The data demonstrated that there was only a slight tendency for
214 the unconjugated liposomes to agglomerate over a 3 month period. In keeping with previous
215 observations on other types of particles [25, 26], particle sizes were larger in HUVEC medium as
216 compared to water or PBS. The HUVEC medium contains serum proteins of different sizes, which
217 may cause agglomeration of liposomes or affect the readings in the Nanosight analysis.
218 No endotoxin contamination ($\leq$ 0.25 EU/ml) was detected for the peptide-conjugated liposomes.
219
220 *Cytotoxicity*
221 The peptide-conjugated liposomes induced cytotoxicity in a log-linear manner (fig. 3). The
222 activated HUVECs displayed the lowest level of cytotoxicity, followed by non-activated HUVECs
223 and co-cultures of HUVECs and macrophages. Nevertheless, there was no difference in the
224 cytotoxicity profile between the different peptide-conjugated liposomes in any of the cell culture
225 models at the concentrations investigated. For all other experiments than the WST-1 assay we used
226 a maximal concentration close to cytotoxicity (cytokine secretion; 40 $\mu g/cm^2$) or a sub-cytotoxic
227 concentration (attachment/uptake; 15.6 $\mu g/cm^2$)
228
229 *Inflammatory response*

The concentration of MIP-1α, TNF-α, IL6, ICAM-1, VCAM-1, G-CSF and IL1-β were measured in the supernatants of cell cultures after 24 hr exposure to the liposomes (figs. 4 and 5). A 24 h exposure for assessment of the inflammation response was used as earlier time points, i.e. 2 and 6 hr, might be too early for production and detection of cytokine and cell adhesion molecule secretion in the cell culture supernatant. In general, the secretion of inflammatory cytokines by non-activated HUVECs was relatively low. These cytokines were slightly increased in the supernatant of activated HUVECs (3-4 fold as compared to non-activated HUVECs). The co-cultures of HUVECs and macrophages had substantially higher concentrations of cytokines in the supernatants (up to 100-fold), which are most likely originating from the macrophages. Importantly, the exposure to peptide-conjugated liposomes did not affect the secretion of cytokines in any of the cell culture models.

Targeting and uptake of liposomes by HUVECs

The targeting and internalization of the peptide-conjugated liposomes were assessed by combination of flow cytometry, fluorescent microscopy and fluorometric determination of the concentration of peptide-liposome conjugates in the supernatant in the three cell cultures. Figure 6 depicts a semi-quantitative analysis of the concentration of conjugates that remained in the supernatant of the cell cultures after 2 or 6 hr exposure. This particular experimental setup cannot distinguish between peptide-conjugated liposomes that have been internalized or merely attached to the cell membrane. Nevertheless, there was higher attachment or uptake of peptide-conjugated liposomes at 6 hr as compared to 2 hr. Liposomes with peptide **2** displayed the highest level of attachment or uptake in activated HUVECs. Liposomes with peptide **2** or **3** also displayed higher attachment or uptake in the co-culture as compared to liposomes with peptide **1**.

In an attempt to distinguish between attachment and uptake of liposomes in HUVECs and macrophages, we used fluorescence microscopy (fig. 7). However, it was inherently difficult to obtain clear two-dimensional images of co-cultures because HUVECs are flat on the surface of the microscope slide, whereas macrophages are round cells that attach on top of the endothelial cells. The fluorescent microscopy showed presence of liposomes (red colour in the images) in the perinuclear area of HUVECs after 2 hr exposure, indicating internalization of all types of peptide-conjugated liposomes. Due to the quality of the images it was not possible to quantitatively assess the uptake of peptide-conjugated liposomes. In the co-cultures, both HUVECs and macrophages displayed uptake of peptide-conjugated liposomes, but it was not possible to measure differences in the uptake of liposomes with different peptides or cell types.

A direct quantitative comparison of differences in the adhesion/uptake of peptide-conjugated liposomes was carried out by flow cytometry (fig. 8). This showed that the liposome with peptide **2** had higher fluorescent signal after 2 hr exposure as compared to liposomes with peptide **1** or **3**. It should be noted that the flow cytometry method in this study cannot distinguish between fluorescence signals from outside and inside cells. However, the collective experimental evidence from the fluorometric determination of peptide-liposome conjugates in the supernatant (fig. 6), fluorescence microscopy (fig. 7) and flow cytometry (fig. 8) indicates uptake of peptide-conjugated liposomes in activated HUVECs and THP-1 cells.

Discussion

The results from this study show that HUVECs had either a higher uptake of liposomes with peptide **2** as compared to liposomes with peptide **1** and **3**, or a faster uptake within the exposure period. Moreover, the targeting of peptide **2** was also observed in co-cultures of HUVECs and macrophages, indicating a somewhat robust delivery of the peptide-conjugated liposomes to

HUVECs in a pro-inflammatory environment where scavenging by activated macrophages may lead to inefficient targeting. The targeting of HUVECs with these peptide-conjugated cationic liposomes did not cause cytotoxicity or inflammation.

The present study allows for a direct comparison between two peptide-conjugated liposomes for targeting integrins under the exact same experimental conditions. It has been shown that HUVECs have a higher expression of integrin α5β1 than αvβ3 and αvβ5, whereas other types of endothelial cells have a different expression of integrins [15, 16]. Both peptide **2** and **3** target the integrin αvβ5, indicating the difference between these peptides is mainly that peptide **3** has a competing binding to integrin αvβ3 (i.e. vitronectin receptor) and α5β1 (i.e. fibronectin receptor). The difference between peptide **2** and **3** suggests that the internationalization of peptide-conjugated liposomes via binding to the fibronectin receptor could be more effective than the vitronectin receptor. It is also possible that the affinity for binding to the specific receptors is higher for peptide **2** in comparison to peptide **3**. It should be noted that the peptides were lipidated for anchorage to liposomes. This type of hydrophobic association is less strong or stable than covalent bonding. However, detachment of the lipidated peptides from liposomes is chemically unfavourable in the aqueous environment of cell cultures.

During an inflammatory response, circulating human leukocytes are able to leave the blood and enter the site of injury by migration through endothelial and sub-endothelial matrices. The integrins on endothelial cells are heavily involved in this migration process together with cell adhesion molecules PECAM (CD31), ICAM-1, VCAM-1 and selectins [27]. Our results indicated enhanced binding/uptake of peptide-conjugated liposomes in activated HUVECs. It has been described that TNF-α activated HUVECs had increased uptake of anti-VCAM-Fab'-conjugated liposomes as compared to non-activated cells [28]. Other studies on IL-1α activated HUVECs also showed enhanced uptake of liposomes that were coupled with antibodies against cell adhesion molecules on

endothelial cells [29, 30]. Activated macrophages engulf materials through binding to scavenger receptors. Thus, activated macrophages may compete with endothelial cells with regards to uptake of peptide-modified liposomes. In addition, there was uptake of liposomes with peptide **2** in HUVECs in the co-culture with activated macrophages. Liposomes with peptide **3** only showed uptake in the co-culture, indicating that it was mainly engulfment by macrophages. These reflections stem from observations in cultured cells, which is test system utilised in this study.. One of the on-going issues in nanomedicine is the effect of protein corona [31]. In this study the cell culture medium contain serum which is required for cell survival and growth.. Thus, the peptide-conjugated liposomes were most likely coated with a protein corona. Interestingly, it has been shown that a protein corona favours the internalization of liposomes by macrophages and tumour cells. [32]. Circulating nanoparticles in the blood stream are most likely coated with a protein corona, but selective targeting has been shown for cell adhesion molecule-conjugated liposomes that accumulated in the target tissue in a rat experimental model of myocardial infarction [33].

It has been previously demonstrated that administration of cationic liposomes may lead to activation of the innate immune system typified by the instigation of the complement system and strong myeloid inflammatory cell response [34, 35]. Moreover, at high dosage *in vivo*, cationic micelles have been demonstrated to cause inflammatory gene expression in lung, spleen and the liver, although there was no sign of toxicity with respect to routine pathology, histology and clinical chemistry end-points [36]. This cationic charge has also been shown to be associated with increased cellular toxicity [37, 38]. There is strong evidence that exposure to some types of carbon-based and metaloxide nanomaterials can increase the expression of ICAM-1 and VCAM-1 on the cell membrane of HUVECs [39-41]. This activation can lead to attachment between HUVECs and macrophages; the latter can be stimulated to accumulate lipids in the presence of carbon-based nanomaterials [42-44]. In this study a comprehensive investigation of potential adverse effects

following exposure of HUVECs and macrophages showed that the peptide-conjugated liposomes did not induce secretion of cell adhesion proteins (i.e. ICAM-1 and VCAM-1) or inflammatory response after 24 hr exposure at concentrations where adhesion/uptake to HUVECs were observed after 2 and 6 hr. However, it should also be emphasized that endothelial cells may not receive the highest exposure *in vivo* because large quantities of intravenously administrated particles accumulate in the liver [45].

In summary, the data here demonstrated liposomes modified with peptide **2** to be a promising targeting candidate for improved specific delivery to activated endothelial cells on the lumen-side of atherosclerotic plaques in the arterial tree. Liposomes with peptide **2** have potential for delivery of a payload of therapeutic and/or imaging agents to application site while sparing or at least minimising exposure of normal healthy tissues.

Despite being positively charged, the peptide-conjugated liposomes had negligible adverse immunogenic capacity and low-level of cytotoxicity to activated HUVECs and macrophages.

Acknowledgements

This work was supported by Lundbeck Foundation Center for Biomembranes in Nanomedicine (grant no: 0602-02331B), Danish Council of Independent Research (Medical Sciences, grant no. 12-126262) and Villum Fonden (Center for Biomolecular Nanoscale Engineering). The authors declare no conflicts of interest.

References

1. Narayanaswamy R, Wang T, Torchilin VP. Improving peptide applications using nanotechnology. Curr Topics Med Chem 2016; **16:** 253-270.

2. Li F, Jin L, He L, Deng Y, He NY. Nanoparticles applied for therapy and diagnosis in common diseases. Sci Adv Materials 2015; **7:** 2103-2122.

3. Scott RC, Crabbe D, Krynska B, Ansari R, Kiani MF. Aiming for the heart: targeted delivery of drugs to diseased cardiac tissue. Expert Opinion on Drug Delivery 2008; **5:** 459-470.

4. Bowey K., Tanguay JF, Tabrizian M. Liposome technology for cardiovascular disease treatment and diagnosis. Expert Opinion in Drug Delivery 2012; **9:** 249-265.

5. Hosta-Rigau L, Schattling P, Teo BM, Lynge ME, Stadler B. Recent progress of liposomes in nanomedicine. J Materials Chem B 2014; **2:** 6686-6691.

6. Xu LQ, Yu H, Yin SP, Zhang RX, Zhou YD, Li J. Liposome-based delivery systems for ginsenoside Rh2: *in vitro* and *in vivo* comparisons. J Nanopart Res 2015; **10:** 415.

7. Zhang ZP, Mei L, Feng SS. Paclitaxel drug delivery systems. Expert Opinion on Research delivery 2013; **10:** 325-340.

8. Lestini BJ, Sagnella SM, Xu Z, Shive MS, Richter NJ, Jayaseharan J, Case AJ, Kotte-Marchant K, Anderson JM, Marchant RE. Surface modification of liposomes for selective cell targeting in cardiovascular drug delivery. Journal of Controlled Release 2002; **78:** 235-247.

9. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nature Reviews Drug Discovery 2005; **4:** 145-160.

10. Ng EWM, Shima DT, Calias P, Cunningham ET, Guyer DR, Adamis AP. Pegaptanib, a targeted anti-VEGF for ocular vascular disease. Nature Reviews Drug Discovery 2006; **5:** 123-132.

11. Farjo KM, Ma JX. The potential of nanomedicine therapies to treat neovascular disease in the retina. Journal of Angiogenesis Research 2010; **2:** 21.

12. Gilad Y, Noy E, Senderowitz H, Albeck A, Firer MA, Gellerman G. Synthesis, biological studies and molecular dynamics of new anticancer RGD-based peptide conjugates for targeted drug delivery. Bioorganic and Medicinal Chemistry 2016; **24:** 294-303.

13. Hamano N, Negishi Y, Fujisawa A, Manandhar M, Sato H, Katagiri F, Nomizu M, Aramaki Y. Modification of the C16Y peptide on nanoparticles is an effective approach to target endothelial and cancer cells via the integrin receptor. Int J Pharmaceutics 2012; **428:** 114-117.

14. Mekuria SL, Debele TA, Chou HY, Tsia HC. IL-6 antibody and RGD peptide conjugated Poly(amidoamine) dendrimer for targeted drug delivery of HeLa cells. The Journal of Physical Chemistry B 2016; **120:** 123-130.

15. Short SM, Talbott GA, Juliano RL. Integrin-mediated signaling events in human endothelial cells. Mol Biol Cell 1998; **9:** 1969-1980.

16. Baranska P, Jerczynska H, Pawlowska Z, Koziolkiewicz W, Cierniewski C. Expression of integrins and adhesive properties of human endothelial cell line EA.hy 926. Cancer Genomics Proteomics 2005; **2:** 265-270.

17. Weis SM, Cheresh DA. αv integrins in angiogenesis and cancer. Cold Spring Harbour Perspectives in Medicine 2011; **1:** a006478.

18. Caccavari F, Valdembri D, Sandri C, Bussolino F, Serini G. Integrin signaling and lung cancer. Cell Adhesion and Migration 2010; **4:** 124-129.

19. Li R, Luo M, Ren M, Chen N, Xia J, Deng X, Zeng M, Yan K, Luo T, Wu J. Vitronectin regulation of vascular endothelial growth factor-mediated angiogenesis. Journal of Vascular Research 2014; **51:** 110-117.

20. Sroka IC, Anderson TA, McDaniel KM, Nagle RB, Gretzer MB, Cress AE. The laminin binding integrin α6β1 in prostate cancer perineural invasion. Journal of Cellular Physiology 2010; **224:** 283-288.

21. Veevers-Lowe J, Ball SG, Shuttleworth A, Kielty CM. Mesenchymal stem cell migration is regulated by fibronectin through α5β1-integrin-mediated activation of PDGFR-β and potentiation of growth factor signals. Journal of Cell Science 2011; **124:** 1288-1300.

22. Shi Q, Wang J, Wang XL, VandeBerg JL. Comparative analysis of vascular endothelial cell activation by TNF-alpha and LPS in humans and baboons. Cell Biochemistry and Biophysics 2004; **40:** 289-303.

23. Klingberg H, Loft S, Oddershede LB, Møller P. The influence of flow, shear stress and adhesion molecule targeting on gold nanoparticle uptake in human endothelial cells. Nanoscale 2015; **7:** 11409-11419.

24. Klingberg H, Oddershede LB, Loeschner K, Larsen EH, Loft S, Møller P. Uptake of gold nanoparticles in primary human endothelial cells. Toxicol Res 2015; **4:** 655-666.

25. Hemmingsen JG, Møller P, Nojgaard JK, Roursgaard M, Loft S. Oxidative stress, genotoxicity, and vascular cell adhesion molecule expression in cells exposed to particulate matter from combustion of conventional diesel and methyl ester biodiesel blends. Environ Sci Technol 2011; **45:** 8545-8551.

26. Frikke-Schmidt H, Roursgaard M, Lykkesfeldt J, Loft S, Nøjgaard JK, Møller P. Effect of vitamin C and iron chelation on diesel exhaust particle and carbon black induced oxidative damage and cell adhesion molecule expression in human endothelial cells. Toxicol Lett 2011; **203:** 181-189.

27. Seguin J, Nicolazzi C, Mignet N, Scherman D, Chabot GG. Vascular density and endothelial cell expression of integrin alpha v beta 3 and E-selectin in murine tumours. Tumour Biology 2012; **33:** 1709-1717.

28. Kang DI, Lee S, Lee JT, Sung BJ, Yoon JY, Kim JK, Chung J, Lim SJ. Preparation and in vitro evaluation of anti-VCAM-1-Fab'-conjugated liposomes for the targeted delivery of the poorly water-soluble drug celecoxib. J Microencapsul. 2011; **28**: 220-7.

29. Gunawan RC, Auguste DT. The role of antibody synergy and membrane fluidity in the vascular targeting of immunoliposomes. Biomaterials. 2010; 31: 900-7.

30. Gunawan RC, Auguste DT. Immunoliposomes that target endothelium in vitro are dependent on lipid raft formation. Mol Pharm. 2010; **7**: 1569-75.

31. Caracciolo, G. Liposome–protein corona in a physiological environment: Challenges and opportunities for targeted delivery of nanomedicines. Nanomedicine: Nanotechnology, Biology, and Medicine. 2015; 11; 543–557.

32. Corbo C, Molinaro R, Taraballi F, Toledano Furman NE, Sherman MB, Parodi A, Salvatore F, Tasciotti E. Effects of the protein corona on liposome-liposome and liposome-cell interactions. Int J Nanomedicine. 2016; **11**: 3049-63.

33. Scott RC, Wang B, Nallamothu R, Pattillo CB, Perez-Liz G, Issekutz A, Del Valle L, Wood GC, Kiani MF. Targeted delivery of antibody conjugated liposomal drug carriers to rat myocardial infarction. Biotechnol Bioeng. 2007; **96**: 795-802.

34. Battaglia L, Gallarate M. Lipid nanoparticles: state of the art, new preparation methods and challenges in drug delivery. Expert Opinions in Drug Delivery 2012; **9:** 497-508.

35. Bozzuto G, Molinar A. Liposomes as nanomedical devices. International Journal of Nanomedicine 2015; **10:** 975-999.

36. Knudsen KB, Northeved H, Kumar PE, Permin A, Gjetting T, Andersen TL, Larsen S, Wegener KM, Lykkesfeldt J, Jantzen K, Loft S, Møller P, Roursgaard M. In vivo toxicity of catotonic micelles and liposomes. Nanomedicine 2015; **11:** 467-477.

37. Iwaoka S, Nakamura T, Takano S, Tsuchiya S, Aramaki Y. Cationic liposomes induce apoptosis through p38 MAP kinase-caspase-8 Bid pathway in macrophage-like RAW264.7 cells. Journal of Leukocyte Biology 2006; **79:** 184-191.

38. Lv H, Zhang S, Wang S, Cui S, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. Journal of Controlled Release 2006; **114:** 100-109.

39. Mikkelsen L, Jensen KA, Koponen IK, Saber AT, Wallin H, Loft S, Vogel U, Møller P. Cytotoxicity, oxidative stress and expression of adhesion molecules in human umbilical vein endothelial cells exposed to dust from paints with or without nanoparticles. Nanotoxicology 2013; **7:** 117-134.

40. Vesterdal LK, Mikkelsen L, Folkmann JK, Sheykhzade M, Cao Y, Roursgaard M, Loft S, Møller P. Carbon black nanoparticles and vascular dysfunction in cultured endothelial cells and artery segments. Toxicol Lett 2012; **214:** 19-26.

41. Forchhammer L, Loft S, Roursgaard M, Cao Y, Riddervold IS, Sigsgaard T, Møller P. Expression of adhesion molecules, monocyte interactions and oxidative stress in human endothelial cells exposed to wood smoke and diesel exhaust particulate matter. Toxicol Lett 2012; **209:** 121-128.

42. Cao Y, Jacobsen NR, Danielsen PH, Lenz AG, Stoeger T, Loft S, Wallin H, Roursgaard M, Mikkelsen L, Møller P. Vascular effects of multi-walled carbon nanotubes in dyslipidemic ApoE-/- mice and cultured endothelial cells. Toxicol Sci 2014; **138:** 104-116.

43. Cao Y, Roursgaard M, Danielsen PH, Møller P, Loft S. Carbon black nanoparticles promote endothelial activation and lipid accumulation in macrophages independently of intracellular ROS production. PLoS One 2014; **9:** e106711.

44. Cao Y, Roursgaard M, Jacobsen NR, Møller P, Loft S. Monocyte adhesion induced by multi-walled carbon nanotubes and palmitic acid in endothelial cells and alveolar-endothelial co-cultures. Nanotoxicology 2016; **10:** 235-244.

45. Kermanizadeh A, Balharry D, Wallin H, Loft S, Møller P. Nanomaterial translocation - the biokinetics, tissue accumulation, toxicity and fate of materials in secondary organs - a review. Critical Reviews in Toxicology 2015; **45:** 837-872.

Legends to figures

Fig 1. Schematic representation of the applied synthesis strategy for the selected peptides: 1. Fmoc deprotection of the resin followed by SPPS. 2. Selective Alloc deprotection. 3. Lipidation of the Lys residue via amide coupling 4. Fmoc deprotection of the N-terminal. 5. Fluorescent tagging of the N-terminal by amide coupling of ATTO465. 6. Deprotection and release of the final peptide product.

Fig 2. The peptides utilised for targeting of activated endothelial cells. The regions important for the targeting and recognition of receptors on the cells are highlighted in red (DFKLFAVYIKYR for peptide **2** and RGD for peptide **3**). Peptide **1** contains a scrambled sequence of amino acids without specific targeting to receptors on endothelial cells.

Fig 3. Cytotoxicity of peptide-conjugated liposomes in non-activated HUVECs (a), activated HUVECs (b) or co-cultures of HUVECs and macrophages (c). The cells were exposed to cell medium (C) or peptide-conjugated liposomes for 24 hr. The graphs show results of liposomes with peptide **1** (blue, diamonds), **2** (red, squares) or **3** (green, triangles). The cytotoxicity measured via WST-1 assay. The values depict the mean ± SEM (n=3).

Fig 4. Cytokine secretion following exposure to peptide-conjugated liposomes. The cells were exposed to cell medium as the negative control (Cont) or increasing concentrations of the peptide-conjugated liposomes for 24 hr. The bars show results of liposomes with peptide **1** (blue), **2** (red) or **3** (green). Missing bars are due to undetectable concentrations of cytokines. The values represent the mean ± SEM (n=3).

Fig 5. Secretion of ICAM-1 and VCAM-1 following exposure to peptide-conjugated liposomes. The cells were exposed to cell medium as the negative control (Cont) or increasing concentrations of the peptide-conjugated liposomes for 24 hr. The bars show results of liposomes with peptide **1** (blue), **2** (red) or **3** (green). Missing bars are due to undetectable concentrations of cell adhesion molecules. The values represent the mean ± SEM (n=3).

Fig 6. Uptake of peptide-conjugated liposomes by non-activated HUVECs, activated HUVECs and a co-culture of macrophages and HUVECs. The cells were exposed to (15.6 μg/cm^2) of the peptide-modified liposomes for 2 and 6 hr before the supernatant was removed, transferred to a fresh plate and fluorescence measured. For the control, the peptide-conjugated liposomes were incubated in empty wells before the above procedure was repeated. The percentage is the ration between fluorescence in the cell culture supernatant and fluorescence in the same volume of peptide-conjugated liposome suspension without cells. The values represent the mean ± SEM (n=3) with significance indicated by *P<0.05 and **P<0.005 as compared to the liposomes modified with peptide **1** at the particular time-point.

Fig 7. Fluorescent microscopy images of liposome uptake following 2 hr exposure to liposomes with peptide **1** (a, b), **2** (c, d) or **3** (e, f) (15.6 μg/cm^2) to activated HUVECs (a, c, e) or co-culture of macrophages and HUVECs (b, d, f) (scale bar - 25 μm). Red spots (i.e. liposomes) can be seen in the perinuclear region of HUVECs, whereas it is not possible to discern differences in the uptake of different liposomes. Macrophages are visible on top of the HUVECs. Uptake of peptide-conjugated liposomes in macrophages is difficult to see in these images because they are focussed on HUVECs, which gives the round macrophages a bright light from the curved cell membrane.

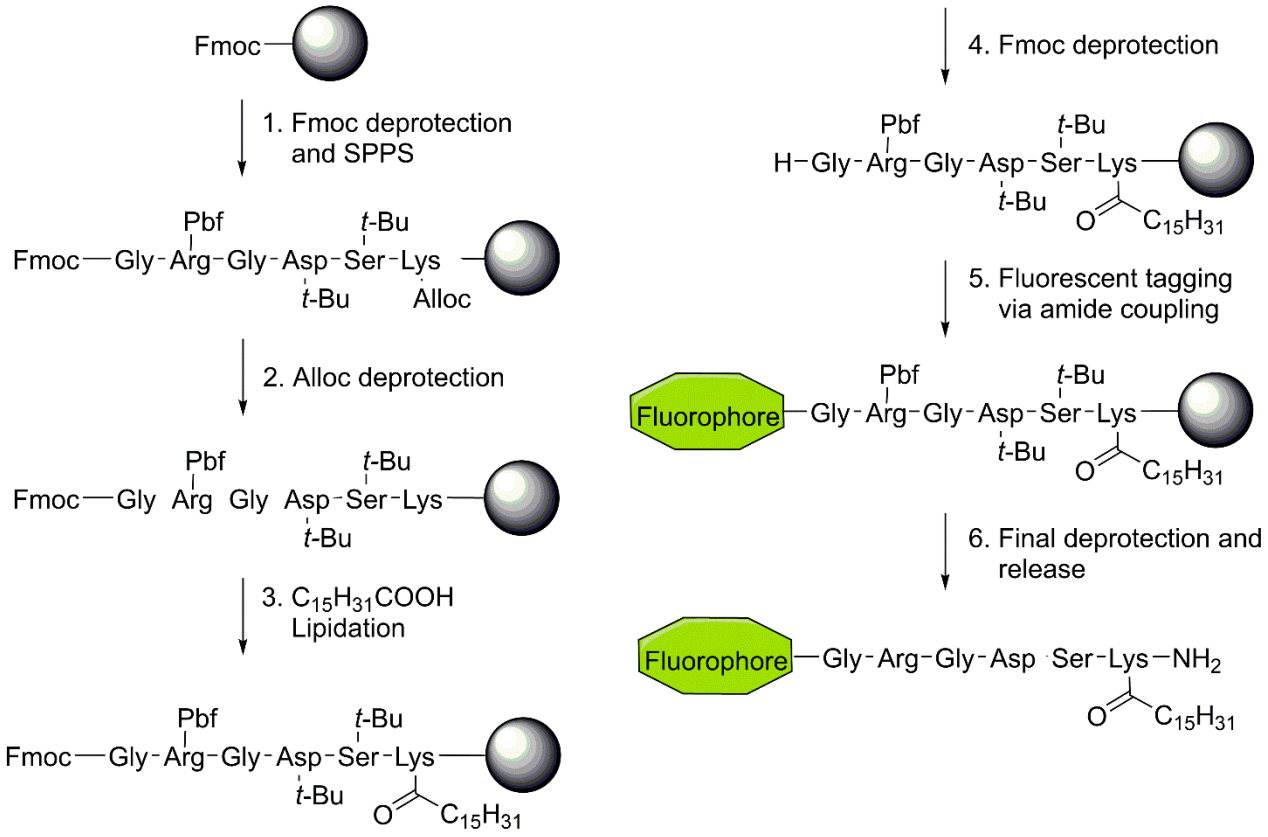
Fig 8. Flow cytometric analysis of cell-associated fluorescence. Activated HUVECs were incubated with 15.6 μg/cm^2 of liposomes with peptide **1** (black), **2** (red) or **3** (blue) for 2 hr. The values represent mean fluorescence from 10000 cells per treatment ± SEM (n=3), significance indicated by * = $p<0.05$ as compared to the liposomes modified with peptide **1**. The insert shows an example of the flow cytometry analysis with the number of cells (y-axis) as the function of fluorescence in the cell (x-axis). A total of 10^4 cells have been analysed for each peptide-conjugated liposome.

Table 1. Particle size of unconjugated liposomes freshly prepared (measured within 2 hr of extrusion) or after 3 months incubation at 4°C after the extrusion step.

	Liposome size of fresh samples			**Liposome size after 3 months**	
	Mean (nm)	**Mode (nm)**	**Zeta potential (mV)**	**Mean (nm)**	**Mode (nm)**
Filtered water	73.7 ± 11.4 (3)	55.7 ± 7 (3)	ND	95.6 ± 20.7 (3)	74.6 ± 18.6 (3)
PBS	117.3 ± 16.7 (6)	107.1 ± 21.8 (6)	21.9 (1)	133.7 ± 31.4 (3)	128.4 ± 21.7 (3)
Complete HUVEC medium	204.6 ± 37.4 (6)	203 ± 41.2 (6)	ND	254.1 ± 59.1(3)	216.6 ± 23.5 (3)

The Nanosight analysis measured particle sizes in suspensions in the range between 10 and 1000 nm. The "mean" is the average particle size of the suspension, whereas the "mode" is the size of the most abundant particle size. The results are mean and SEM (number of independent experiments). The Zeta potential is the charge of the particles. ND: not determined.

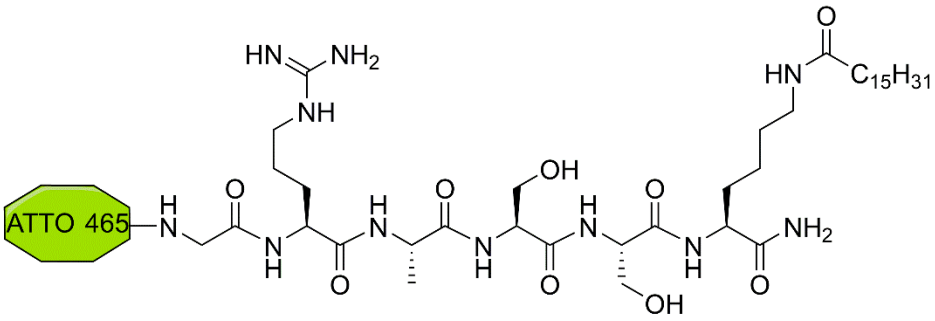
580 Fig.1.



581

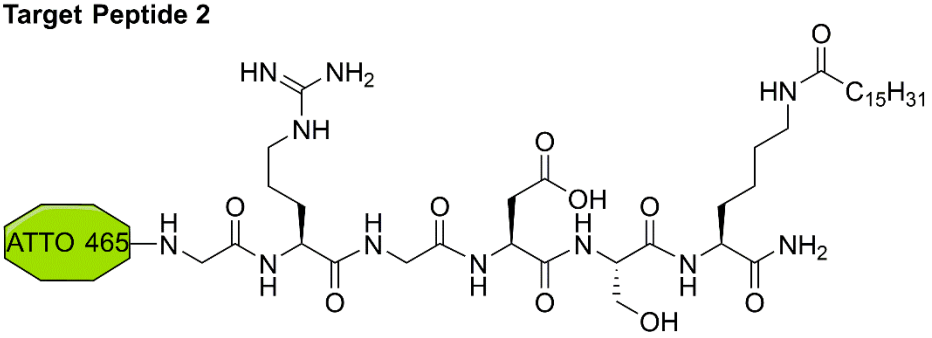
582

583 Fig. 2.



Target Peptide 1: ATTO465 - Gly - Arg - Ala - Ser - Ser - Lys(Palm) - NH_2

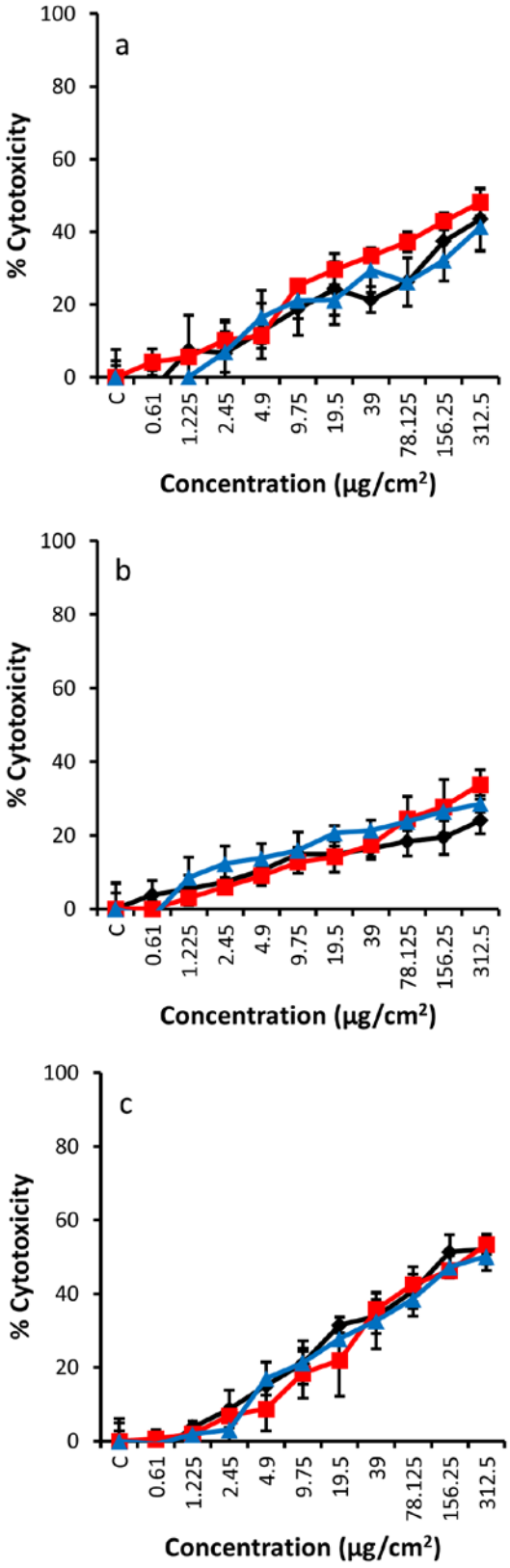
Target Peptide 2



584 **Target Peptide 3**: ATTO465 - Gly - **Arg** - **Gly** - **Asp** - Ser - Lys(Palm) - NH_2

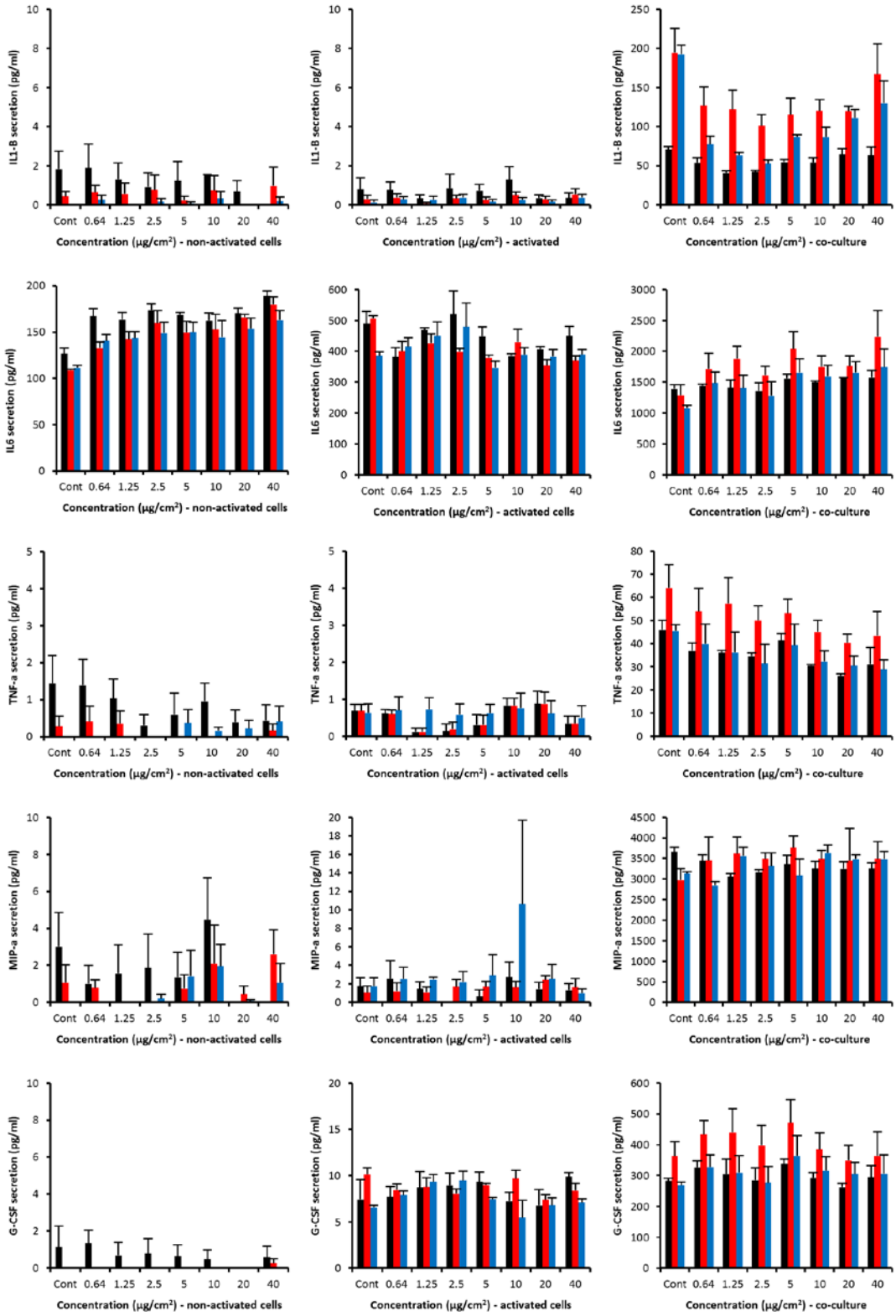
585

586 Fig. 3.


a
b
c
% Cytotoxicity
100
80
60
40
20
0
C
0.61
1.225
2.45
4.9
9.75
19.5
39
78.125
156.25
312.5
Concentration (µg/cm²)

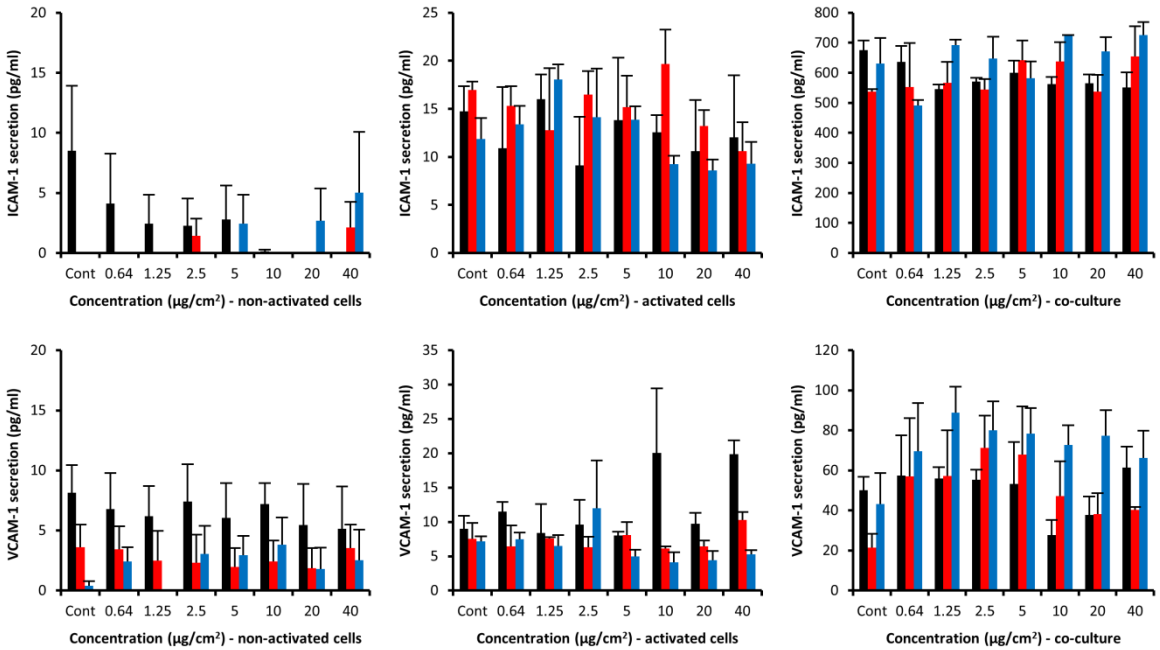
587

588 Fig. 4.


IL1-B secretion (pg/ml)
Concentration (μg/cm²) - non-activated cells
Concentration (μg/cm²) - activated
Concentration (μg/cm²) - co-culture
IL6 secretion (pg/ml)
Concentration (μg/cm²) - non-activated cells
Concentration (μg/cm²) - activated cells
Concentration (μg/cm²) - co-culture
TNF-a secretion (pg/ml)
Concentration (μg/cm²) - non-activated cells
Concentration (μg/cm²) - activated cells
Concentration (μg/cm²) - co-culture
MIP-a secretion (pg/ml)
Concentration (μg/cm²) - non-activated cells
Concentration (μg/cm²) - activated cells
Concentration (μg/cm²) - co-culture
G-CSF secretion (pg/ml)
Concentration (μg/cm²) - non-activated cells
Concentration (μg/cm²) - activated cells
Concentration (μg/cm²) - co-culture
Cont 0.64 1.25 2.5 5 10 20 40

589

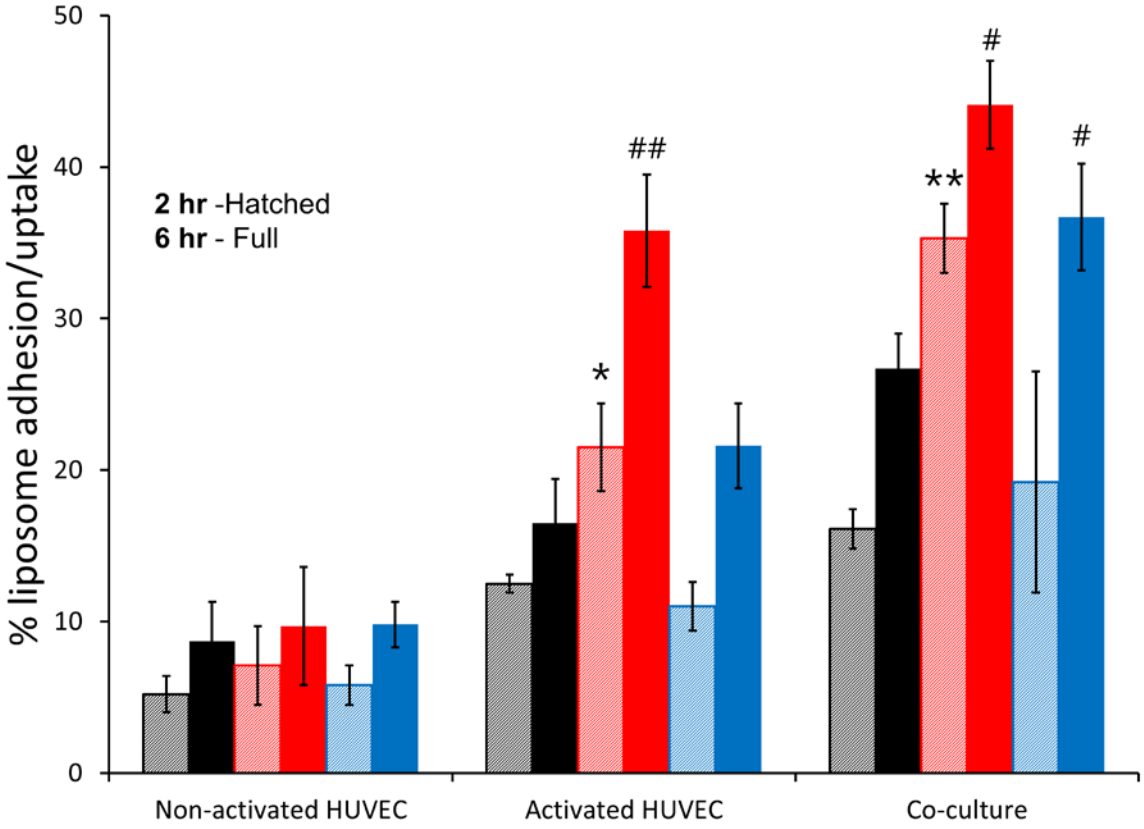
590 Fig. 5.



591

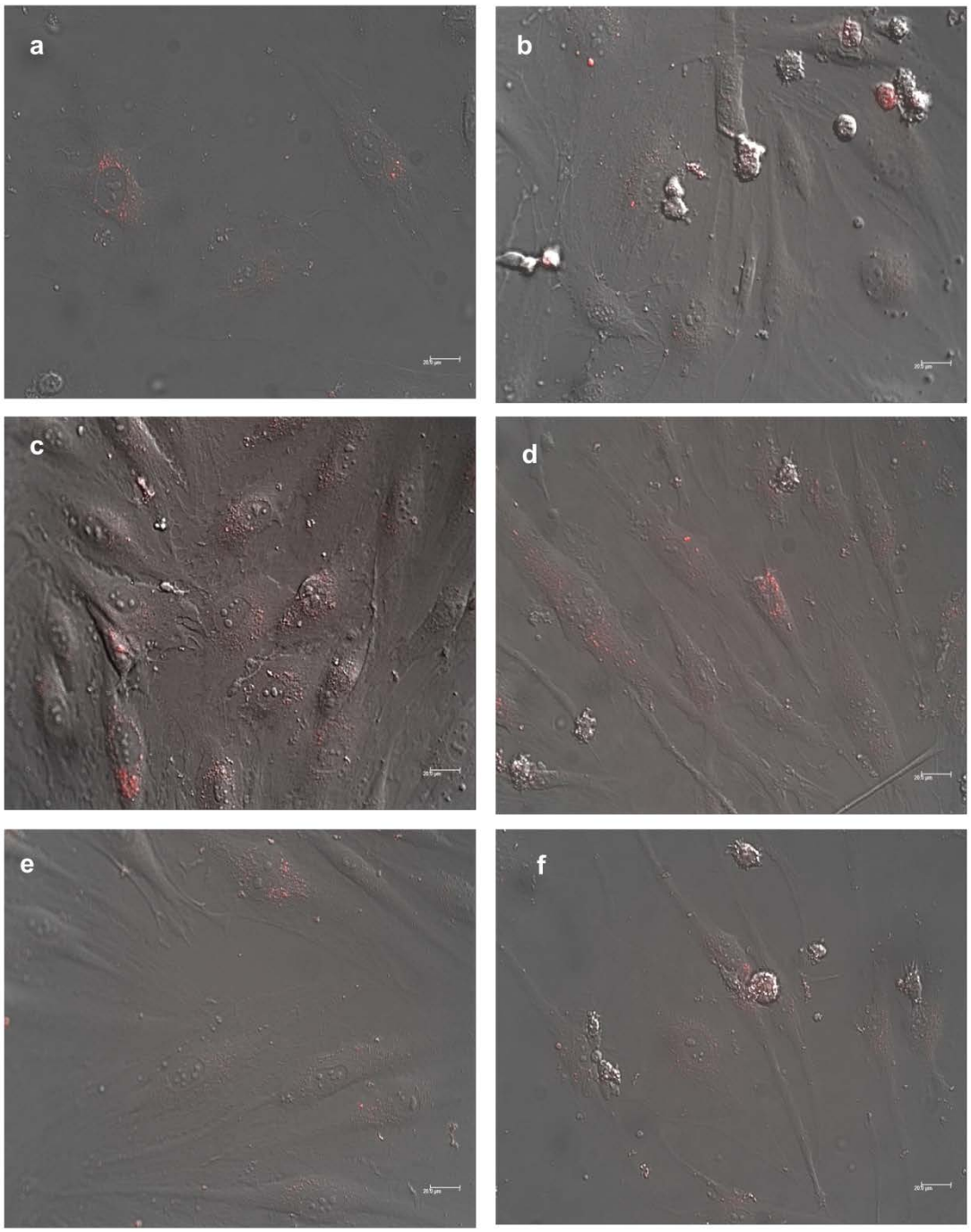
592

593 Fig. 6.


50
40
30
20
10
0
% liposome adhesion/uptake
2 hr -Hatched
6 hr - Full
*
##
**
#
#
Non-activated HUVEC
Activated HUVEC
Co-culture

594

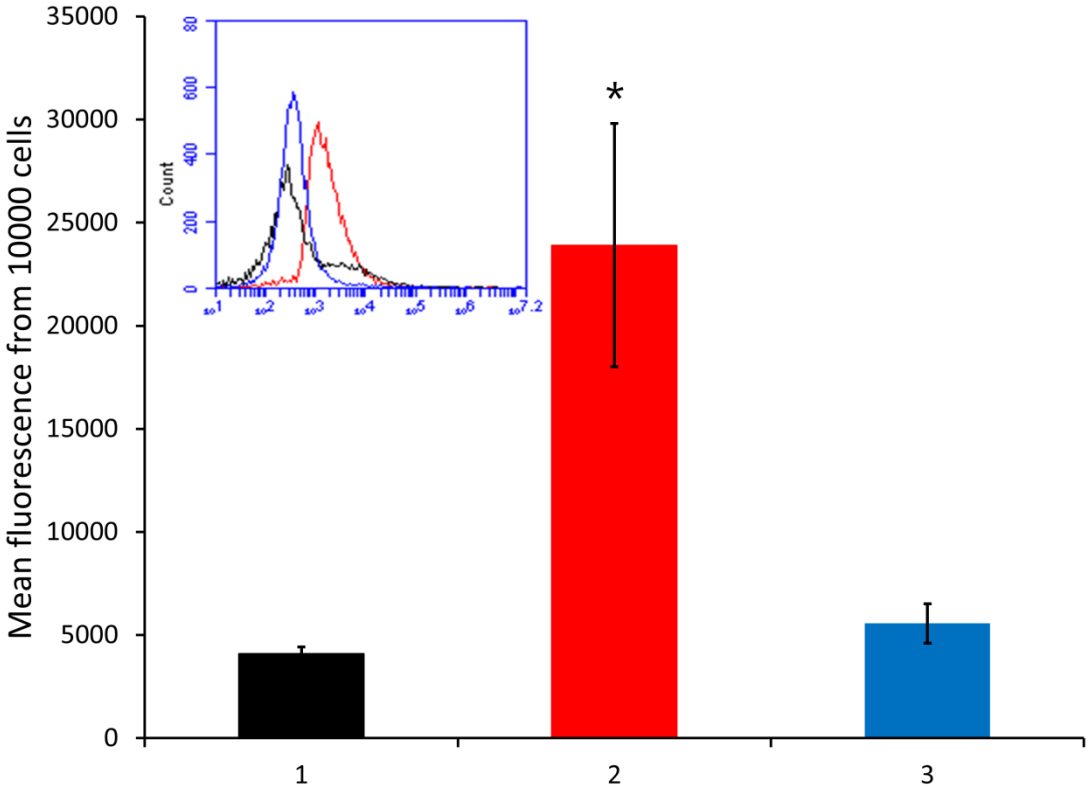
595 Fig. 7.



596

597

598 Fig. 8.


Mean fluorescence from 10000 cells
35000
30000
25000
20000
15000
10000
5000
0
1
2
3
*
Count
0
200
400
600
800

599